



Sheep (*Ovis aries*) airway epithelial cells support ovine herpesvirus 2 lytic replication *in vivo*

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ABSTRACT

Ovine herpesvirus 2 (OvHV-2) is the causative agent of sheep-associated malignant catarrhal fever (SA-MCF), a frequently fatal disease of some members of the order Artiodactyla. OvHV-2 is carried as a lifelong subclinical infection in sheep (*Ovis aries*). To date OvHV-2 has not been propagated *in vitro* and this has hampered studies of viral pathogenesis and efforts to develop a vaccine to protect animals from SA-MCF. Lytic OvHV-2 replication occurs in the lungs of experimentally infected sheep at early times post-inoculation (PI) and in the nasal cavities of naturally infected sheep during virus shedding episodes. Identification of specific cell types supporting lytic virus replication *in vivo* provides information that can be used in the development of an *in vitro* propagation system for the virus. Using fluorescence immunohistochemical techniques, we identified lytically infected alveolar epithelial cells in the lungs of sheep early during infection. Lytically infected epithelial cells were also detected in samples of nasal secretions collected from naturally infected sheep during episodes of virus shedding. This is the first reported identification in the natural reservoir species of specific cell types that support OvHV-2 lytic replication *in vivo*.

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1. Introduction

Ovine herpes virus 2 (OvHV-2), a gammaherpesvirus in the genus *Macavirus*, establishes a lifelong subclinical infection in domestic sheep (*Ovis aries*) (Davison et al., 2009; Plowright, 1990). However, transmission of OvHV-2 from sheep to susceptible members of the order Artiodactyla, such as North American bison (*Bison bison*) and cattle (*Bos taurus*), can result in the disease called malignant catarrhal fever (MCF); MCF caused by infection with OvHV-2 is referred to as sheep-associated (SA)-MCF (Plowright, 1990). SA-MCF outbreaks result in severe losses to the commercial bison industry (Berezowski et al.,

2005; Li et al., 2006; O'Toole et al., 2002; Schultheiss et al., 2000). Since no vaccine is available to protect clinically susceptible species from disease, control of MCF relies on keeping these species separated from sheep. However, biosecurity measures alone are not sufficient to prevent SA-MCF outbreaks, particularly in light of the fact that OvHV-2 has been transmitted from sheep to bison over long distances (Li et al., 2008b).

The inability to propagate OvHV-2 *in vitro* has constrained studies of OvHV-2 pathogenesis which have largely been limited to examination of naturally occurring cases of SA-MCF or SA-MCF experimentally induced using lymphoblastoid cells persistently infected with OvHV-2 (reviewed in Ackermann, 2006; Russell et al., 2009). The development of an experimental animal infection system using nasal secretions containing infectious cell-free OvHV-2 has allowed us to begin investigating host–virus

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interactions using the natural route of infection under controlled conditions (Gailbreath et al., 2008; O'Toole et al., 2007; Taus et al., 2006, 2005). Continued progress in SA-MCF studies requires the development of an *in vitro* system to propagate and manipulate OvHV-2. We sought to make use of the natural virus carriers, sheep, to provide insight into the requirements for OvHV-2 propagation by identifying cells *in vivo* that are susceptible to infection with OvHV-2 and permissive for lytic virus replication.

In previous studies an RT-PCR assay was used to detect transcripts from open reading frame (ORF) 25, which encodes the major capsid protein of OvHV-2, in order to identify virus replication sites *in vivo* (Cunha et al., 2008; Li et al., 2008a). Examination of RNA extracted from cells contained in nasal secretions and homogenates of turbinates collected from sheep experiencing shedding episodes contained ORF25 transcripts (Cunha et al., 2008). ORF25 transcripts were also detected in lung samples of experimentally infected sheep between three and nine days post-inoculation (PI) (Li et al., 2008a). In order to identify the specific cell types supporting this lytic virus replication *in vivo* an ORF25 protein-specific rabbit antiserum was produced and used in conjunction with antibodies directed against cellular markers to immunolabel lytically infected cells in sheep lungs and nasal cavity cells.

2. Materials and methods

2.1. Animals and experimental infection

Details of the experimental infection of sheep, sample collection and real time PCR and RT-PCR assays have been published (Cunha et al., 2008; Li et al., 2008a). All animal experimental procedures were conducted in accordance with an animal use protocol approved by the Washington State University Institutional Animal Care and Use Committee. Briefly, OvHV-2 uninfected sheep were inoculated with aerosolized nasal secretions containing 1×10^7 genome copies of OvHV-2 or secretions from an uninfected sheep. The sheep were euthanized in pairs on days 1, 3, 5, 7, and 9 PI. Lung samples from day 7 PI had the highest copy numbers of OvHV-2 DNA and all lung samples tested, from cranial, middle, and caudal lobes, contained ORF25 transcripts, therefore lung samples from this time point were selected for use in this study.

2.2. Antiserum production

A purified bacterial recombinant protein was used to immunize three rabbits to produce an antiserum against the ORF25-encoded major capsid protein of OvHV-2. The EcoRI fragment from plasmid Ov728, which contains OvHV-2 DNA from nt 50,721–52,514 (Taus et al., 2007) (DQ198083), was ligated into the EcoRI site of pGEX-4T-3 (Amersham Biosciences) to generate a plasmid encoding predicted ORF25 protein amino acids 658–1256 in frame with glutathione-S-transferase (GST) at the N-terminus. The fusion junction was sequenced to ensure that the open reading frame of ORF25 was maintained (data not shown).

Recombinant protein was induced by the addition of isopropyl- β -D-galactoside (0.8 mM) to the bacterial cul-

ture for 4 h at 30 °C and soluble protein was affinity purified, essentially as described by Frangioni and Neel (1993), using Glutathione Sepharose 4B (Pharmacia Biotech). The purified protein was subjected to acrylamide gel electrophoresis and examined using Coomassie Blue staining. The full length GSTORF25 fusion protein was approximately 95,000 MW, which is in agreement with the predicted size of the ORF25 protein fragment fused to GST (94,200 MW). Thrombin protease was used according to manufacturer's directions (Amersham Biosciences) to cleave the 26,000 MW GST moiety from the ORF25 portion of the protein, predicted to be approximately 68,200 MW. Acrylamide gel electrophoresis of the total cleaved protein followed by staining with Coomassie Blue revealed the expected 26,000 MW protein, a prominent protein of ~54,000 MW and several smaller bands. The ~54,000 MW band was cut from the gel, examined using liquid chromatography and tandem mass spectrometry, and confirmed to be the ORF25 protein (data not shown). Evaluation of the predicted GSTORF25 amino acid sequence using PoPS (World Scientific) revealed potential thrombin cleavage sites within the ORF25 protein which could explain why the ORF25 protein fragment was smaller than predicted.

For immunization of rabbits, the 54,000 MW ORF25 protein fragment was electroeluted from gel slices and emulsified with TiterMax Classic adjuvant (Sigma). Each rabbit was immunized three times with ~10 μ g of protein each time. The priming dose was divided between intramuscular injection in the thigh and subcutaneous injection between the shoulder blades. Specificity of immunized rabbit sera was assessed using immunoblotting. Pre- and post-immunization sera were reacted against total cell lysates prepared from bacteria expressing the recombinant fusion protein or GST only. Immune sera from all three rabbits recognized the full length GSTORF25 fusion protein; pre-immune sera did not react with the fusion protein. Additionally, the sera were reacted with purified full length GSTORF25 protein and thrombin cleaved protein. All three rabbit immune sera recognized both the full length and cleaved ORF25 protein. Antiserum from one of the immunized rabbits was selected for further use in immunostaining experiments.

2.3. Immunostaining

2.3.1. Lung sections

Formalin fixed paraffin-embedded lung samples were collected from experimentally infected sheep (day 7 PI) and a negative control sheep (Li et al., 2008a). Thin (3 μ m) sections mounted on glass slides were deparaffinized in Clear-Rite™ 3 (Richard-Allen Scientific), rehydrated through graded alcohol to water, and rinsed in phosphate buffered saline (PBS). Slides were then subjected to 20 min antigen retrieval in a steam decloaker containing citrate buffer (pH 6.0; Dako). After cooling, a Super PAP Pen HT™ (Research Products International Corp.) was used to draw a hydrophobic margin around thin sections to retain fluid reagents on slides. Thereafter slides were placed in a humidified chamber protected from direct light and maintained at room temperature.

Table 1
Antibodies used for immunostaining infected sheep lung.

Antibody	Specificity	Reactivity	Dilution	Source	Co-localization with ORF25 protein
Anti-ORF25 (rabbit)	ORF25	OvHV-2 infected cells	1:200	This paper	na ^a
MCA2538H (mouse)	CD79a	B cells	1:20	AbD Serotec	N
DH59b (mouse)	CD172a	Monocytes, granulocytes	5 µg/ml	VMRD	N
MCA835G (mouse)	WC1	γ-δ T cells	5 µg/ml	AbD Serotec	N
MCA1853 (mouse)	CD163	Tissue macrophages	1:200	AbD Serotec	N
AE1/AE3 (mouse)	Cytokeratin	Epithelial cells	1:50	Dako	Y
proSP-C (rabbit)	Surfactant protein C	Type II alveolar epithelial cells	1:100–200	Millipore	Y

^a na—not applicable.

For standard fluorescence immunohistochemistry, thin sections were first incubated in PBS for 15 min after which non-specific protein binding was blocked for 60 min using 5% goat serum/PBS/0.1% Tween-20. The blocking solution was then replaced and incubated for 2 h with primary antibody (Table 1) diluted in blocking solution. Unbound primary antibody was removed with three 10 min washes in PBS which was followed by a 2 h incubation with secondary antibodies diluted 1:200 (Alexa Fluor[®] 555 or 488 conjugated goat anti-mouse IgG or anti-rabbit IgG; Molecular Probes, Inc.). Unbound secondary antibody was removed with three 10 min washes in PBS after which slides were cover slipped using ProLong[®] Gold antifade mounting media containing DAPI (Molecular Probes, Inc.). Controls included slide incubations without primary antibody or replacement of rabbit antiserum with “pre-immune” serum collected prior to immunization. For dual labeling experiments, mixtures of secondary antibodies were checked for cross-reaction. Non-specific immunoreactivity was not observed in any of these control experiments.

For dual labeling experiments using two rabbit primary antisera, Zenon[®] rabbit IgG labeling kits (Molecular Probes, Inc.) were used as directed by the manufacturer to bind fluorophore labeled Fab fragments (Fab) to anti-surfactant protein-C (Alexa Fluor[®] 555) and anti-ORF25 (Alexa Fluor[®] 488) antisera in Fab: primary antibody ratios of 6:1. Standard immunostaining was applied except as follows: Thin sections were incubated 2 h with Zenon-labeled primary antibodies diluted in PBS/0.2% Triton-X 100. Following three 15-min washes in PBS/0.2% Triton-X 100, thin sections were then post-fixed 15 min in 4% formaldehyde. A final three 5-min washes in PBS was followed by cover slipping. Additional controls included incubation of sequential lung sections from infected sheep with Zenon reagents without primary antibody or Zenon-labeled rabbit pre-immune serum, and incubation of lung sections from a non-infected sheep with labeled primaries. Non-specific immunoreactivity was not observed.

Epi-fluorescence was imaged using an Axio Imager.M1 microscope (Carl Zeiss Microimaging) equipped with an X-Cite 120 FI Illuminating system (EXFO Photonic Solutions), and digitally captured using an AxioCam MRc5 digital camera connected to a desktop computer running AxioVision (version 4.7.1.0). Z-stack images were deconvolved and processed into maximum intensity projections using ImageJ for Windows (version 1.43f) (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997–2009) with the Iterative

Deconvolve 3D plug-in installed (version 5.2) (Dougherty, 2005). Figure images were prepared using Photoshop Elements (version 4.0, Adobe Systems Inc.).

2.3.2. Nasal secretion samples

Nasal secretions were collected daily as described (Li et al., 2004) from 6-month old sheep naturally infected with OvHV-2. Real-time PCR was used to identify sheep which were experiencing a shedding episode (Li et al., 2004) and more secretions were collected from those animals within 4 to 6 h. Secretions were clarified by centrifugation and the cell pellets were resuspended in PBS. Debris, such as hay and dirt, was allowed to settle for 5 min and the supernatant was collected. Cells were pelleted from the supernatant by centrifugation at 500 × g for 5 min and washed two times with PBS. The cells were resuspended in an appropriate volume of PBS and 50–75 µl was added to Probe-On slides (Fisher Scientific) by centrifugation at 1000 rpm for 3 min using a Shandon Cytospin 2 (Thermo Scientific). Cells were fixed in 95% ethanol for 5 min and air dried. Slides were stored at –80 °C with desiccant until use.

For immunostaining slides were incubated in PBS at room temperature for 5 min then blocked for 30 min at room temperature with DakoCytomation protein blocker (Dako). Excess blocker was removed and the cells were incubated for 1 h at room temperature with anti-cytokeratin mouse monoclonal antibody cocktail AE1/AE3 (Vector) diluted 1:50 in blocking buffer (PBS/5% horse serum/0.1% Tween-20). Secondary antibody for the mouse antibody was Oregon Green 488-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). Following incubation with secondary antibody, slides were washed and then incubated with the rabbit anti-OvHV-2 major capsid protein antiserum diluted 1:400 in blocking buffer for 2 h at 37°C. The slides were washed in PBS and incubated with Alexa Fluor[®] 594-conjugated goat anti-rabbit IgG (Invitrogen) diluted 1:500 for 30 min at room temperature. The slides were rinsed in PBS followed by double distilled water and mounted in Vectashield (Vector Laboratories). Fluorescent labeling was visualized with a Zeiss Axioscope 2 Plus and recorded using AxioVision 3.0 software.

3. Results

3.1. Detection of OvHV-2 major capsid protein in alveolar epithelial cells

As represented in Fig. 1, immunoreactivity (green fluorescence) mediated by the anti-ORF25 protein-specific

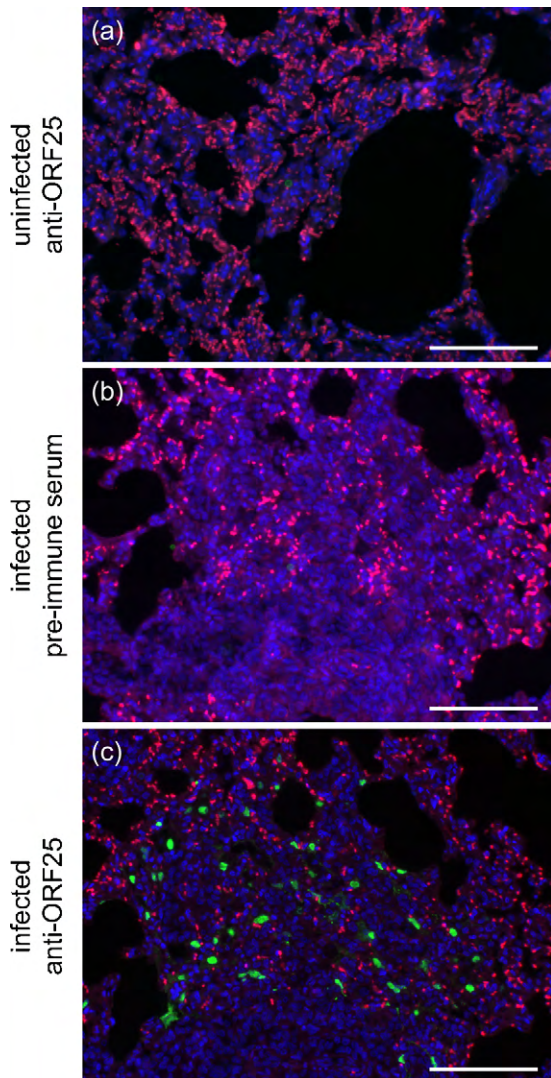


Fig. 1. Detection of OvHV-2 major capsid protein in the lung of sheep at seven days post-inoculation. No immunoreactivity is observed when (a) anti-ORF25 serum is incubated with a lung section from an uninfected sheep, or (b) when pre-immune serum is incubated with a lung section from an infected sheep. Only autofluorescence (red) and DAPI-stained nuclei (blue) are observed in these sections. (c) Anti-ORF25 immunoreactivity (green) is observed in the replicate lung section to (b). Scale bars = 100 μ m.

rabbit serum was sensitive and specific in fixed thin sections of lung after antigen retrieval. Tissue architecture was equally referenced between each image by red autofluorescence; particularly evident are autofluorescing erythrocytes. Immunoreactivity (green) was observed within sections of lung from infected sheep (7 dPI) incubated with the ORF25 protein-specific antiserum (Fig. 1c) but was not observed within the same lesion of a replicate thin section incubated with pre-immune serum (Fig. 1b). In addition, immunoreactivity was not observed when similarly treated lung sections from non-infected sheep were incubated with ORF25 protein-specific antiserum. Within the lung of infected sheep, ORF25 immunor-

eactivity was generally most intense within the nucleus of fluorescing cells, which was sometimes accompanied by less intense labeling within the cytoplasm (Fig. 1c). This distribution of the ORF25 protein is consistent with nuclear capsid assembly as occurs in herpesviruses (Henson et al., 2009). OvHV-2 infected cells occurred as scattered, focal areas in infected lungs. Some sections had one or two foci of infected cells while viral protein was not detected on other sections from the same sample (data not shown).

Most often the ORF25 protein expressing cells were in areas that appeared to be air spaces that had been infiltrated with cells (Fig. 2b). Examination of replicate lung sections stained with hematoxylin and eosin showed that the foci consisted of groups of alveoli filled with macrophages and fewer lymphocytes (Fig. 2a and c). Septa of affected alveoli were also expanded by infiltrates of mononuclear cells. The inflammatory cells were occasionally associated with mild necrosis resulting in disruption of alveolar septa and replacement by macrophages mixed with small amounts of cellular debris and a few neutrophils. The remainder of the lung tissue was normal. In the previous report describing the histopathologic findings from the lungs of the sheep that were also used in this study, only one sheep was found to have rare focal areas of mild interstitial and alveolar inflammation (Li et al., 2008a). This is consistent with the results reported here in that infected cell foci were not present in every section of lung examined and therefore many of the lung sections examined previously most likely did not contain detectable lesions. A few small foci consisting of a few macrophages and lymphocytes filling alveoli or expanding septa were within sections from OvHV-2 negative sheep but were much less frequent and much milder than the foci in OvHV-2 positive sheep (data not shown). The location and morphology of the virus positive single labeled cells suggested that epithelial and lymphoid cells were possible cell types infected with OvHV-2. To determine the identity of the OvHV-2 infected cells a series of antibodies directed against various cellular proteins (Table 1) was used in co-labeling experiments with the ORF25 protein-specific antiserum. Fig. 2b shows co-labeling of cells with anti-cytokeratin antibodies and the ORF25 protein antiserum. Cytokeratins are abundant cytoskeletal proteins expressed in epithelial cells (Upasani et al., 2004). Most, greater than 90%, of the cells positive for ORF25 protein expression were also cytokeratin positive, indicating that epithelial cells of the lower respiratory tract support lytic OvHV-2 replication. Examination of multiple sections of lung from two sheep at seven days PI failed to show co-labeling of viral protein in cells labeled with markers for lymphoid cells (Table 1).

The mammalian lower respiratory tract is lined with a low cuboidal epithelium in the bronchioles. The respiratory epithelium terminates in the alveoli which are lined by highly specialized type I and type II alveolar epithelial cells (AECs) (Grubor et al., 2006). Because the lung samples had been fixed in formalin at the time of necropsy, the architecture of the lung had been preserved. Therefore it was possible to exclude the epithelial cells of the conducting airways as the cells expressing ORF25 protein.

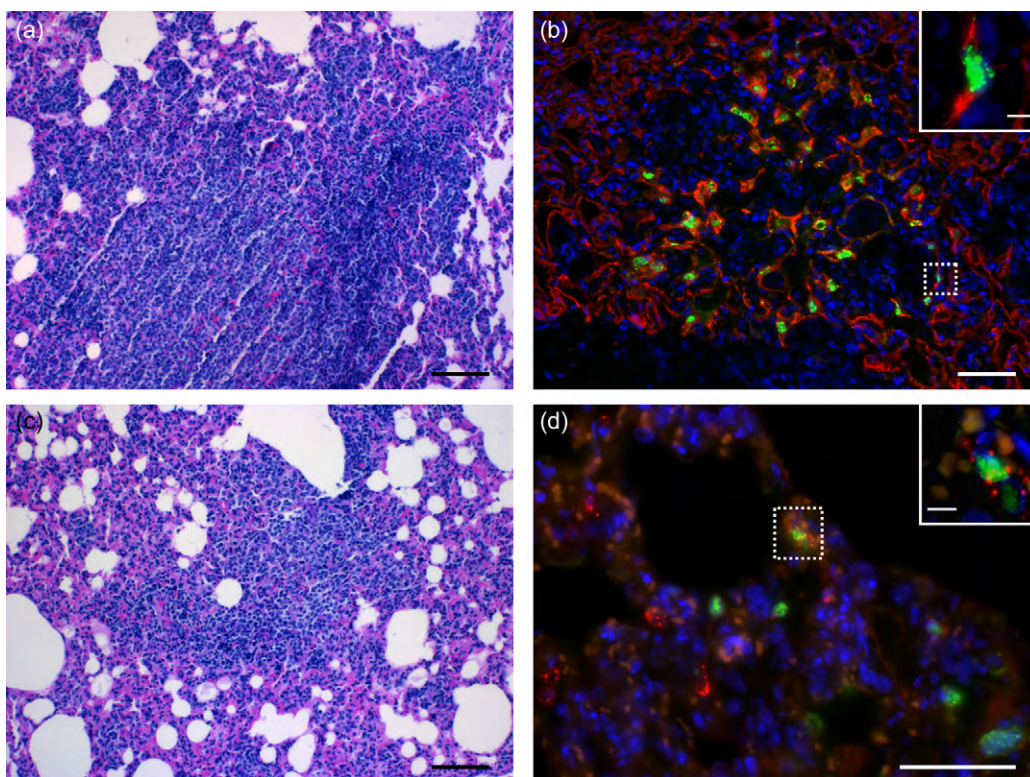


Fig. 2. Co-localization of OvHV-2 major capsid protein with pneumocyte marker antibodies, cytokeratin (top row) and surfactant protein C (bottom row) in the lung of sheep at seven days post-inoculation. (a and c) Low magnification views of H & E stained sections of infected lung tissue which are replicate sections to those used in the fluorescence images shown in the right column panels ((b and d), respectively). Shown are lung sections from infected sheep dually immunoreactive for anti-ORF25 (green) and (b) anti-cytokeratin (red) or (d) anti-surfactant protein-C (red). In both sets of images, DAPI-stained nuclei appear blue and insets each represent a maximum intensity projection of a deconvolved z-stack from the respective regions indicated by white-dashed boxes. In these examples note that immunoreactivity for OvHV-2 major capsid protein is observed in cells expressing cytokeratin and surfactant protein C. Autofluorescence in panels b and d appears much less intense compared to Fig. 1 and is orange-yellow. Autofluorescence intensity in all thin sections was considerably less than fluorescence mediated by primary antibody when present in the same fluorescence channel. Scale bars: (a and c) 100 μ m; (b and d) 50 μ m; (b and d insets) 5 μ m.

We decided to use antibody directed against surfactant protein C (SP-C) to determine whether the OvHV-2 infected cells were type II AECs. SP-C is considered to be a specific marker for type II AECs (Rooney et al., 1994).

In order to co-label lung sections with the SP-C and ORF25 protein antisera, which are both polyclonal rabbit antisera, Zenon[®] labeling was used to directly conjugate different fluorophores to each. Fig. 2d shows that some ORF25 protein positive cells were also positive for SP-C, indicating that type II AECs support lytic OvHV-2 replication. Interestingly, the number of SP-C expressing cells decreased around the foci of OvHV-2 infected cells and the center of the foci had no cells with visible SP-C expression (Fig. 2d), which was in contrast to cytokeratin expression which was detected within the foci of infected cells (Fig. 2b). Although the changes noted in SP-C expression are qualitative, they were consistently observed in multiple sections of tissue.

3.2. Detection of ORF25 protein in nasal cavity epithelial cells

Nasal secretions of naturally infected sheep experiencing virus shedding episodes contain cell-free infectious

OvHV-2 and cells expressing ORF25 transcripts (Cunha et al., 2008; Kim et al., 2003; Taus et al., 2005). We sought to determine the specific cell type(s) supporting lytic virus replication during virus shedding following reactivation. Examination of the cells using a modified Romanowski stain (Diff-Quik[®]) showed numerous epithelial cells as well as some mononuclear and polymorphonuclear cells (Fig. 3a). Immunostaining using the anti-cytokeratin antibodies (Fig. 3b) and ORF25 protein-specific antiserum (Fig. 3c) showed that epithelial cells of the nasal cavity were lytically infected with OvHV-2 (Fig. 3d). Nasal cells collected from uninfected sheep showed labeling for cytokeratin, but no signal for the ORF25 protein (data not shown).

4. Discussion

OvHV-2 has not been isolated *in vitro*; attempts to isolate virus from tissues of SA-MCF affected animals were unsuccessful (Huck et al., 1961; Clark et al., 1972). The lack of an *in vitro* culture system for OvHV-2 has limited the types of studies that can be conducted to study OvHV-2 replication. Therefore this study was designed to take

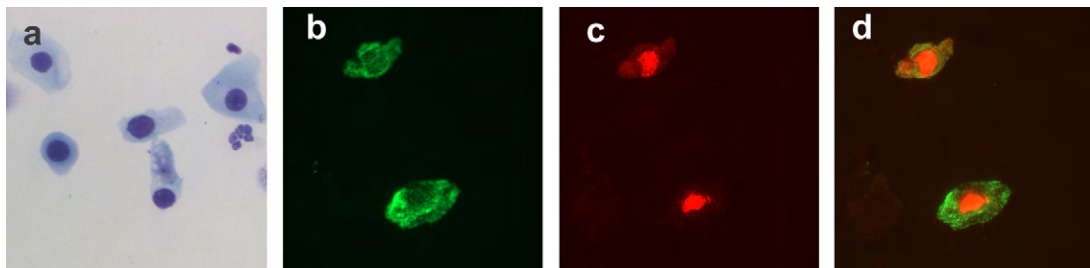


Fig. 3. Detection of OvHV-2 major capsid protein in epithelial cells in nasal secretions of naturally infected high shedder sheep. Cytospin preparations of nasal secretion cells collected from naturally infected sheep experiencing intense virus shedding events were (a) stained with a Romanowski stain or (b) labeled with a mouse anti-cytokeratin monoclonal antibody cocktail (green) and (c) a rabbit ORF25 protein-specific (red). (d) Same cells shown in (b and c) with immunoreactivity visualized using a dual fluorescence filter.

advantage of the experimental animal infection model we have developed (Taus et al., 2005) in order to identify specific cell types supporting OvHV-2 lytic replication *in vivo* and thus gain knowledge about potential requirements for *in vitro* culture of the virus.

Natural transmission of OvHV-2 from latently infected sheep to naïve animals is predominantly through the shedding of virus in nasal secretions (Li et al., 2004). Experimental aerosol inoculation of cell-free OvHV-2 has resulted in reproducible infection of sheep, bison, and rabbits (Gailbreath et al., 2008; O'Toole et al., 2007; Taus et al., 2005). Therefore, although experimental aerosol infection is not exactly the same as natural infection, it has key features of natural infection i.e. the infectious OvHV-2 is cell free and the route of infection is the respiratory tract. It is plausible then, that the lytically infected type II AECs identified in this work are among the cells initially targeted by OvHV-2. Moreover, intravenous and intraperitoneal inoculation of sheep and rabbits, respectively, with cell-free OvHV-2 did not result in infection (Gailbreath et al., 2008; Li et al., 2008a). This suggests that cell-free virus does not efficiently infect peripheral blood mononuclear cells or peritoneal cells such as macrophages and may require replication in the respiratory tract to establish a latent infection in lymphocytes.

In addition to type II AECs, cytokeratin positive/SP-C negative cells were also found to be infected. These could be type I AECs, which do not express SP-C, or they could be type II cells that no longer express SP-C due to OvHV-2 inhibition of cellular gene expression. Gammaherpesviruses are known to inhibit cellular gene expression through the activity of the viral alkaline exonuclease, predicted to be encoded by ORF37 in OvHV-2 (Covarrubias et al., 2009; Hart et al., 2007; Taus et al., 2007).

Occasionally, OvHV-2 infected cells negative for cytokeratin expression were also identified in lung sections. These cells may be non-epithelial or may be epithelial cells in which lytic replication has proceeded to the point that the cytoskeleton has been disrupted. ORF25 protein expression was not detected in macrophages, labeled using a monoclonal antibody directed against CD163, in monocytes or granulocytes, labeled using anti-CD172a antibody, nor in B-cells labeled with anti-CD79a antibody (Table 1). Lack of an ORF25 protein signal in these cell types does not exclude the possibility that one or more of these cell types supported lytic replication; it is possible that the

level of ORF25 protein expression was too low to be detected.

Attempts to label dendritic cells and T-cells were unsuccessful, so it is possible that the cytokeratin and SP-C double negative lytically infected cells were either or both of these cell types. Dendritic cell infection, lytic and latent, with murine herpesvirus-4 (MuHV-4), also known as mouse gammaherpesvirus (MHV68), has been demonstrated following intranasal inoculation (Flano et al., 2000; Hochreiter et al., 2007). T-lymphocytes of naturally infected sheep carry latent OvHV-2 (Li, unpublished data; Meier-Trummer et al., 2010) and we expect that to be the case in experimentally infected sheep as well. Since this study focused on OvHV-2 lytic replication, identification of cell types latently infected with OvHV-2 during early infection was not pursued.

Other OvHV-2 lytic cycle proteins, a tegument protein and the capsid portal protein, have been detected in epithelial and M-cells in the appendices of rabbits with experimentally induced MCF (Meier-Trummer et al., 2009). There are a number of differences between that study and the work reported here. The model used by Meier-Trummer and colleagues involves intravenous inoculation of OvHV-2 infected lymphoblastoid cells versus aerosol inoculation of cell-free virus in this report. Additionally, the previous study focused on OvHV-2 protein expression late in infection after the development of clinical disease whereas the current work examined an early time point after infection in the natural reservoir species. Therefore, it is not possible to directly compare the two studies but it is interesting to note that lytically infected epithelial cells were identified in both.

In order for OvHV-2 to be shed in nasal secretions latent OvHV-2 needs to reactivate, lytically replicate to produce virions and cross the epithelial cell barrier in the respiratory tract. ORF25 transcripts have been detected in turbinate samples and nasal secretion cell pellets from sheep experiencing shedding episodes (Cunha et al., 2008). Examination of nasal cavity cells of shedding sheep revealed ORF25 protein expression in epithelial cells. We propose that reactivated OvHV-2 from latently infected lymphocytes infects epithelial cells in the turbinates during virus shedding (exit) events.

We have previously proposed that OvHV-2 potentially switches cell tropism without specifying cell types in the turbinates and lungs that are susceptible and permissive to

lytic replication (Li et al., 2008a). In this study we demonstrated that *in vivo* different types of epithelial cells are permissive for OvHV-2 lytic replication during virus exit and entry. This suggests to us that cell-free virus found in nasal secretions and lymphocyte derived virus may use different combinations of cellular receptors and viral proteins in order to infect epithelial cells found in lungs and turbinates. Cell tropism switching involving different combinations of viral and cellular proteins has been described for Epstein–Barr virus (EBV) *in vitro* (Chesnokova et al., 2009 and references therein). The next steps in translating the observations from this study will be to examine *in vitro* combinations of cell-free virus and various epithelial cells as well as co-culture of latently infected lymphocytes from sheep with epithelial cells.

Conflict of interest statement

The authors declare no conflicts of interest.

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